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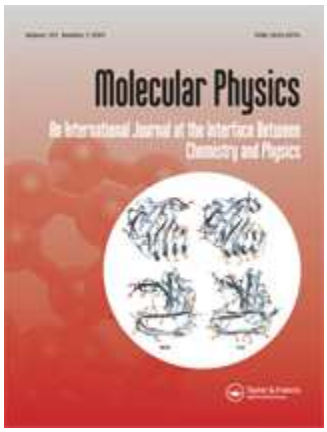

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Effect of pH on superoxide/ hydroperoxyl radical trapping by nitrones: an EPR/kinetic study

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The pH dependence of the apparent rate constant k_{Tap} for $O_2^{\cdot-}/HO_2^{\cdot}$ trapping by four nitrones was examined. In each case, kinetic curves were obtained after treatment of EPR spectra of the spin adduct formed using both singular value decomposition and pseudo-inverse deconvolution methods. Modelling these curves led to evaluate k_{Tap} at various pH values. Analysis of the pH dependence of k_{Tap} permitted the determination of the rate constants for the spin trapping of $O_2^{\cdot-}$ and of $HO_2^{\cdot}$ separately. Whatever the nitrone, our results clearly show that the EPR signals of the nitrone/superoxide spin adducts observed in aqueous media were essentially due to the trapping of the protonated species $HO_2^{\cdot}$

Keywords: EPR; Kinetics; pH Effect; Spin Trapping; Superoxide; Nitrones

1 Introduction

Since the EPR/spin trapping technique was first introduced [1-3], it has found numerous applications in the detection of free radicals produced in chemistry, biology or medicine. It has been notably employed for detecting superoxide in aqueous media using nitrone spin traps [4-6]. For this purpose, a nitrone must trap rapidly this radical and must lead to a long-lived spin adduct. Several reliable methods have been described to determine k_D, the rate constant for the nitrone/superoxide spin adduct decay [7-13]. In contrast, evaluating the apparent rate of superoxide trapping by a nitrone, k_{Tap}, is more problematic and major disagreements exist regarding the data published in this field [11,14-21]. The most popular method used in these studies involves a competition between the nitrone and a superoxide scavenger, and implies that the superoxide spontaneous dismutation, the spin adduct decay and the consumption of the competitor during the course of the experiment correspond to negligible events [11,14-17]. Two years ago, we brought evidences of the importance of these three reactions and showed that their omission resulted in significantly overestimating k_{Tap} [22]. Another method based on a competition between the superoxide trapping by the nitrone and the spontaneous dismutation of this radical yielded lower k_{Tap} values []18,19]. This prompted us to elaborate a new kinetic approach to the evaluation of k_{Tap} in buffered media [20,21]. It was also based on a competition between the superoxide trapping and its spontaneous dismutation, and kinetic curves were obtained after treatment of EPR spectra using both singular value decomposition (SVD) and pseudo-inverse deconvolution methods before their computer modelling. Whatever the method used, it is widely admitted that the kinetic parameters related to the spin trapping of superoxide by nitrones are pH dependent. On one hand, nitrone/superoxide spin adducts decayed

more slowly in acidic media [7-9]. On the other hand, k_{Tap} also greatly varied with pH [14,16], owing to the fact that superoxide $O_2^{-\cdot}$ and its protonated form $HO_2^{\cdot}$ were not distinguished in these studies, both radicals leading to the same EPR-detectable spin adduct in aqueous media. The relative concentrations of $HO_2^{\cdot}$ and of $O_2^{-\cdot}$ depend on pH, the pK_A value for the acidic dissociation reaction of the hydroperoxyl radical being equal to 4.88 [23]. Considering that nitrones react differently with $HO_2^{\cdot}$ and $O_2^{-\cdot}$, this justifies the pH dependence of k_{Tap} .

Recently, we have found that three nitrones were particularly efficient for superoxide detection: the ethyl 2-methyl-3,4-dihydro-*2H*-pyrrole-carboxylate *1*-oxide **1** (EMPO, $k_{Tap} \approx 11$ $dm^3mol^{-1}s^{-1}$ at pH 7.2), the diethyl 3,4-dihydro-*2*H-pyrrole-2,2-dicarboxylate *1*-oxide **2** (DEPO, $k_{Tap} \approx 31$ $dm^3mol^{-1}s^{-1}$ at pH 7.2), and 1,3,5-tri [(N- (1-diethylphosphono)-1-methyl-ethyl) -*N*-oxy-aldimine] benzene **3** (TN, $k_{Tap} \approx 9$ $dm^3mol^{-1}s^{-1}$ at pH 7.2) [20,21]. Among them, TN is the only lipophilic trap, with an octanol/water partition coefficient higher than 300 [24]. By far, DEPO was found the fastest at trapping superoxide, but the spin adduct thus formed decayed very rapidly, particularly when high nitrone concentrations were used. However, kinetic experiments with all these traps were only performed at pH 7.2. To proceed with this study, we have examined the pH dependence of the rate constant for the superoxide trapping by EMPO, DEPO, TN and have compared the results obtained to that given by the well-known 2,2-dimethyl-3,4-dihydro-*2H*-pyrrole *1*-oxide **4** (DMPO) (see figure 1). Analysis of the data obtained at various pH permitted to evaluate separately the rate constant for the trapping of $HO_2^{\cdot}$ and $O_2^{-\cdot}$ by the nitrone considered and to determine which species is actually responsible for the formation of the adduct detected by EPR.

[Insert figure 1 about here]

In this whole study, the spin adduct detected by EPR after trapping either $O_2^{-\cdot}$ or $HO_2^{\cdot}$ with a nitrone N in aqueous media will be denoted N-O_2H. With the aim of simplifying the notation, the term 'superoxide' will first be used to designate both superoxide and hydroperoxyl radicals as a whole in the part of this work devoted to the study of the pH effect on k_{Tap}. Similarly, the symbol '$O_2^{-\cdot}$' in the kinetic model [equations (1)-(4)] as well as in the rate equations (5)-(8) will represent either protonated or non-protonated forms of the radical. In a second part of this study, the two species will be considered separately to evaluate each individual contribution to the formation of N-O_2H.

2 Experimentals

2.1 Materials

The nitrones EMPO **1**, DEPO **2** and TN **3** were synthesised and purified according to procedures described previously [24-27]. DMPO **4** (Sigma-Aldrich Co.) was purified by vacuum distillation before use. Diethylenetriaminepentacetic acid (DTPA), 3-carboxy-2,2,5,5-tetramethylpyrrolidin-1-oxyl **5** (3CP), xanthine and xanthine oxidase were obtained from Sigma-Aldrich Co. Buffer solutions, were stirred gently for six hours in the presence of a chelating iminodiacetic acid resin to remove trace metal impurities.

2.2 Achievement of experimental kinetic curves

All experiments were made in 0.1 mol dm^{-3} phosphate buffer (pH range: 6.2-9.3). Superoxide was produced using a xanthine-xanthine oxidase system. In a standard experiment, the medium contained a nitrone N (3-200 mmol. dm^{-3}), 1.6 mmol.dm^{-3} xanthine, 3 mmol.dm^{-3} diethylenetriaminepentacetic acid, 3CP (0.5-1.1 µmol dm^{-3}) used as internal standard, and 0.04 unit.cm^{-3} xanthine oxidase. Air was bubbled into the

medium for one min. before addition of xanthine oxidase. A part of the EPR signal showing at least one line of the superoxide adduct N-O_2H and one line of 3CP spectra was recorded every 42 s. Noise was reduced using the SVD procedure, and the kinetic curves (adduct concentration *vs*. time) were obtained after deconvolution of the signal using the pseudo-inverse method, according to procedures extensively described previously [20]. These calculations were achieved with the help of a home-made computer programme written in FORTRAN, using subroutines given in Numerical Recipes [28]. At each pH value and for each nitrone, three experiments were performed at three different nitrone concentrations, one of which was set high enough to trap out most of the superoxide produced. In the case of DEPO, the procedure was modified as described previously [20,21], because the spin adduct decays too rapidly at high nitrone concentrations. EPR essays were carried out at 20°C in capillary tubes by using a Bruker EMX spectrometer operating at X-band with 100 kHz modulation frequency. General instrument settings were as follows: microwave power, 20 mW; modulation amplitude, 0.1-0.18 mT; receiver gain, 2 10^5 - 2 10^6; time constant, 164 ms; scan time, 21 s; scan width, 1.5 - 6 mT; 2 scans.

2.3 Determination of the apparent rate constant k_{Tap}

At a given pH, the kinetic model considered can be described by equations (1)-(4),

$$X \xrightarrow{k_X} O_2^{\cdot -} \qquad (1)$$

$$2\ O_2^{\cdot -} + 2\ H^+ \xrightarrow{k_{dis}} O_2 + H_2O_2 \qquad (2)$$

$$O_2^{\cdot -} + H^+ + N \xrightarrow{k_{Tap}} N\text{-}O_2H \qquad (3)$$

$$N\text{-}O_2H \xrightarrow{k_D} Y \qquad (4)$$

where k_X and k_D are the rate constants for the generation of superoxide and for the decay of the spin adduct N-O_2H, respectively, Y representing EPR silent products and X an intermediate derived from xanthine [20]. The apparent rate constant for the second-order trapping reaction is denoted k_{Tap}. The rate equations (5)-(8) can be written from this kinetic model:

$$d[X]/dt = -k_X [X] \quad (5)$$

$$d[O_2^{\cdot-}]/dt = k_X [X] - k_{Tap} [N] [O_2^{\cdot-}] - 2 k_{dis} [O_2^{\cdot-}]^2 \quad (6)$$

$$d[N\text{-}O_2H]/dt = k_{Tap} [N] [O_2^{\cdot-}] - k_D [N\text{-}O_2H] \quad (7)$$

$$d[N]/dt = - k_{Tap} [N] [O_2^{\cdot-}] \quad (8)$$

The term 'superoxide' and the symbol '$O_2^{\cdot-}$' designate here the protonated and non-protonated forms of the radical indiscriminately. The apparent rate constant for the trapping reaction by the nitrone N, k_{Tap}, is pH dependent and includes the contribution of both $HO_2^{\cdot}$ and $O_2^{\cdot-}$ trapping. The second order rate constant for the superoxide spontaneous dismutation reaction, k_{dis}, is also pH-dependent. Computer modelling of the kinetic curves obtained was achieved using a home-made programme and according to equations (5)-(8). In these calculations, the nitrone initial concentration is an experimental parameter, the initial concentrations of superoxide and of N-O_2H are equal to zero, and k_{dis} was evaluated as described further. The standard least-square method was applied to fit the experimental curves, yielding the other parameters. Therefore, the three kinetic curves obtained at three different nitrone concentrations were considered jointly and modelled with the same parameter set (except for parameters varying with the nitrone concentration). The values obtained for the initial concentration $[X]_0$ and for the rate constant k_X had no real meaning and only came up as an empirical model of the superoxide generation. Since they may vary with the solutions of either xanthine or

xanthine oxidase used [20], experiments at various nitrone concentrations were performed with exactly the same superoxide generator. The model corresponding to equations (9)-(12), which was first established by Baxendale [29], describes the superoxide spontaneous dismutation in aqueous media. Here, reaction (12) is negligible below pH 9.5.

$$HO_2^{\cdot} \underset{}{\overset{K_A}{\rightleftharpoons}} O_2^{-\cdot} + H^+ \qquad (9)$$

$$2\ HO_2^{\cdot} \xrightarrow{k_1} H_2O_2 + O_2 \qquad (10)$$

$$O_2^{-\cdot} + HO_2^{\cdot} \xrightarrow{k_2} H_2O_2 + O_2 + HO^- \qquad (11)$$

$$2\ O_2^{-\cdot} \xrightarrow{k_3} H_2O_2 + O_2 + 2\ HO^- \qquad (12)$$

The rate constant k_{dis} can be determined at any pH from equations (13)-(14), where pK_A, k_1 and k_2 are equal to 4.88, 76 10^4 $dm^3.mol^{-1}.s^{-1}$ and 85 10^6 $dm^3.mol^{-1}.s^{-1}$, respectively [23].

$$k_{dis} = (k_1 + \chi\ k_2) / (1 + \chi)^2 \qquad (13)$$

$$\chi = K_A / [H^+] = 10^{(pH-pK_A)} \qquad (14)$$

2.3 Determination of the trapping rate constants $k_{HO_2\cdot}$ and $k_{O_2^{-}\cdot}$.

Because nitrones react differently with $O_2^{-\cdot}$ and with $HO_2^{\cdot}$, reactions (15) and (16) should be considered instead of reaction (3) in the kinetic model, $k_{HO_2\cdot}$ and $k_{O_2^{-}\cdot}$ being the rate constants for the trapping of superoxide radical anion ($O_2^{-\cdot}$) and hydroperoxyl radical ($HO_2^{\cdot}$), respectively, by the nitrone N.

$$O_2^{-\cdot} + N + H^+ \xrightarrow{k_{O_2^{-}\cdot}} N\text{-}O_2H \qquad (15)$$

$$HO_2^{\cdot} + N \xrightarrow{k_{HO_2\cdot}} N\text{-}O_2H \qquad (16)$$

According to Finkelstein *et al.* [14], equation. (17) can be written from equations (14)-(16).

$$k_{Tap} = (k_{HO_2\cdot} + \chi\, k_{O_2\cdot -}) / (1 + \chi) \qquad (17)$$

Using the method described in the previous section, k_{Tap} was determined for each nitrone at five pH values ranging from 6.2 to 9.3. The experimental curves thus obtained were modelled using equation (17), which led to $k_{HO_2\cdot}$ and $k_{O_2\cdot -}$.

3 Results and discussion

3.1 Determination of the rate constants at various pH

Experiments were performed in order to evaluate the pH influence on the trapping of superoxide by four nitrones: EMPO **1**, DEPO **2**, TN **3** and DMPO **4**. Whatever the species trapped by the nitrone N ($O_2^{\cdot -}$ or $HO_2^{\cdot}$, both designated here by the term 'superoxide'), only the protonated adduct N-O_2H will be detected by EPR. Actually, the anionic radical species N-O_2^- is not EPR silent, but its concentration in the pH range considered is negligible, considering the high pKa values of hydroperoxides.

The formulae of the stable nitroxide **5** and of the spin adducts **6**-**9** considered in this study have been drawn in figure 2. Before each kinetic experiment, a full EPR spectrum of the medium was recorded. All the spectra thus obtained were analysed using the computer program elaborated by Duling [30], which led to the values listed in table 1 for the hyperfine splitting constants (hfscs) with the nitrogen (a_N), the β-hydrogen ($a_{H\beta}$) and eventually a γ-hydrogen ($a_{H\gamma}$) or a β-phosphorus (a_P) nuclei. These hfscs were not found to vary in the pH range considered and agree with the literature data [4,10,24,27]. In a previous study [24], we have shown that

the mono-nitroxide **8** (i.e. the mono-adduct) was almost exclusively formed at the tri-nitrone TN **3** concentrations used, the proportion of bi-nitroxide being negligible.

[Insert figure 2 and table 1 about here]

The kinetic approach used, based on a competition between the superoxide trapping and its spontaneous dismutation at a given pH value, permits the consideration of the whole kinetic curve of superoxide adduct formation and decay. This method has been extensively described elsewhere and will only be briefly explained here [20]. For each nitrone N considered and at each pH value studied, a part of the EPR spectrum of N-O_2H was recorded as a function of time at various nitrone concentrations, in the presence of an internal reference (see experimental section). In order to illustrate the method employed, an EPR spectrum of the adduct TN-O_2H **8** recorded at pH 6.2 in the presence of the internal standard 3CP is shown in figure 3, the framed part corresponding to the signal range chosen to perform the kinetic experiments. Using both singular value decomposition and pseudo-inverse deconvolution methods, kinetic curves indicating the time dependant changes in the N-O_2H concentration at various pH were achieved. As an example, the curves obtained with the spin trap **3** at pH 6.2 and at pH 9.3 are given in figure 4. Using equations. (5)-(8), computer modelling of the experimental kinetic curves obtained with each nitrone at each pH considered yielded the values of k_D and k_{Tap} listed in table 2.

[Insert figures 3 and 4 and table 2 about here]

It has been shown that the rate constant for the first-order decay of nitrone/superoxide spin adducts increased with pH and with the spin trap concentration [7-9,20,21,30]. Overall, the values listed for k_D in table 2 agree with

these observations, though a few deviations from this general trend can be noticed. These could be explained by considering two main factors. Firstly, EPR signals recorded under certain extreme conditions (e.g. at high pH values) were often very weak. Actually, it would have been impossible to achieve the kinetic curves from these spectra without the help of SVD and deconvolution methods. In this case, k_D determination could be less accurate, as shown for instance by the errors given in table 2. Secondly, the first-order decay of nitrone/superoxide spin adducts in aqueous media are known to depend on several parameters other than pH and nitrone concentration. In particular, minor changes in the superoxide generator (i.e. in solutions of xanthine or of xanthine oxidase) may affect significantly N-O_2H life-time [8,10,20,21]. Considering the number of experiments performed in this study, different solutions of xanthine and of xanthine oxidase have been used, thereby rending k_D evaluation less accurate. The dependence of the decay rate of nitrone/superoxide adducts on various parameters (pH, nitrone concentration, superoxide generating system, etc.) is certainly an interesting problem that would warrant a more thorough study. A better knowledge of the mechanisms responsible for N-O_2H decay in aqueous solutions would permit the design of new spin traps with increased superoxide adduct life-time. However, the purpose of the present work was mainly to scrutinise the effect of pH on the superoxide trapping rate. Note that the three series of spectra at the three different nitrone concentrations, for each nitrone and at each pH, were recorded under exactly the same conditions. Thus, in the kinetic approach used, minor changes in the superoxide generator would not affect k_{Tap} determination.

A fast survey of k_{Tap} values given in table 2 shows that the same nitrone ranking was obtained at each pH considered: DEPO was always found the fastest at trapping superoxide, followed by EMPO, TN and DMPO. Whatever the nitrone, our results confirmed that k_{Tap} diminished at higher pH. This could suggest that the nitrones would trap the hydroperoxyl radical more rapidly than the superoxide radical anion. At this point however, interpretation of the experimental results requires an more advanced analysis, in which contributions of the protonated and non protonated radicals should be separated.

3.2 Analysis of k_{Tap} variation with pH

In this last part of our study, the superoxide radical anion and the protonated hydroperoxyl radical were distinguished. Our purpose was to determine in which extent each of these two species contributes to the N-O_2H EPR signal observed. As mentioned in the experimental section, $k_{O_2^{-\cdot}}$ and $k_{HO_2^{\cdot}}$ are the second-order rate constants for the trapping of $O_2^{-\cdot}$ and of $HO_2^{\cdot}$ by a nitrone N, respectively. Equation (17), which can be rewritten as equation (18), indicates the relation between $k_{O_2^{-\cdot}}$, $k_{HO_2^{\cdot}}$ and k_{Tap}. Equation(19) can be written from equation (18), in which pK_A is equal to 4.88.

$$k_{Tap} = [k_{HO_2^{\cdot}} + 10^{(pH-pK_A)}\, k_{O_2^{-\cdot}}] / [1 + 10^{(pH-pK_A)}] \qquad (18)$$

$$\log k_{Tap} = \log\, [k_{HO_2^{\cdot}} + (pH - 4.88)\, k_{O_2^{-\cdot}}] - pH + 4.88 \qquad (19)$$

Fitting the model described by equation (18) to the experimental points for nitrones **1-4** yielded the values reported in table 3 for the rate constants $k_{O_2^{-\cdot}}$ and $k_{HO_2^{\cdot}}$. . In figure 5, the decimal logarithm of k_{Tap} has been plotted *vs*. pH. The curves have been obtained according to equation (19) and using the rate constant values listed in

table 3, showing for each nitrone a rather good fit between experimental and calculated k_{Tap} values.

[Insert figure 5 about here]

In the particular case of DMPO, $k_{O_2^{-\cdot}}$ was found near nil, considering the error in its determination. This last result could seem in contradiction with that published by Goldstein *et al.* [32], who have found that $k_{O_2^{-\cdot}}$ was equal to 170 ± 40 $dm^3\ mol^{-1}s^{-1}$ for DMPO. However, they obtained this value by measuring the kinetics of $O_2^{-\cdot}$ consumption in the presence of **4** using the pulse radiolysis technique, while we followed the kinetics of formation of **9** using EPR spectroscopy. According to Goldstein *et al.* [32], only a fraction of the superoxide radical anion yields the nitroxide **9**. This explains the discrepancy mentioned above, all the more so since the yield of the reaction: **4** + $O_2^{-\cdot}$ → **9** is unknown. Consequently, the EPR method is obviously more appropriate to kinetic studies of superoxide trapping by nitrones.

Whatever the nitrone, there is always three order of magnitude between $k_{HO_2^{\cdot}}$ and $k_{O_2^{-\cdot}}$ (see table 3). Thus, the trapping of $O_2^{-\cdot}$ does not intervene significantly in the N-O_2H formation until the superoxide concentration is much higher than that of hydroperoxyl radical. For instance, the proportion of the EPR signal of **6** originating from the trapping of $O_2^{-\cdot}$ by EMPO is lower than 1% at pH 6.5, than 2.5% at pH 7 and than 8% at pH 7.5. In the case of DMPO **4**, these values are raised to *ca*. 2.5%, 8% and 25% at pH 6.5, 7 and 7.5 respectively. This means that the EPR signals of the spin adducts N-O_2H observed in aqueous media around neutral pH were almost exclusively due to the trapping of the protonated species $HO_2^{\cdot}$

4 Conclusion

This study confirms that the spin trapping of $O_2^{\cdot-}/HO_2^{\cdot}$ by nitrones is overall more efficient at low pH. Whatever the nitrone N, the formation of N-O_2H is not only faster in acidic media but the decay rate of this spin adduct increases with pH. When it comes to the trapping rate, the same nitrone ranking was obtained at each pH studied: DEPO was found the fastest at trapping superoxide, followed by EMPO, and TN, these three compounds being much more efficient than DMPO. Examining the pH dependence of the kinetics of N-O_2H formation permitted to evaluate separately the rate constant values for the spin trapping of superoxide radical anion and of hydroperoxyl radical by four nitrones. The results obtained with **1-4** clearly showed that the EPR signals of the nitrone/superoxide spin adducts observed in aqueous media were essentially due to the trapping of the protonated species $HO_2^{\cdot}$. For the nitrones tested, $k_{O_2^{\cdot-}}$ was always found lower than 2 dm^3 mol^{-1} s^{-1}. In previous studies conducted with ten commonly used nitrones, **1-3** were found the most efficient for superoxide/hydroperoxyl radical trapping at neutral pH [20-21]. Thus, this very low reactivity of nitrones with the superoxide radical anion should be considered as a serious drawback, precluding the use of these compounds in quantitative studies of $O_2^{\cdot-}/HO_2^{\cdot}$ formation in aqueous media. Despite this, compounds **1-3** could still find interesting applications in qualitative studies in water or biological media. Therefore, the development of new and more efficient traps is still a necessity, in particular for superoxide radical anion detection.

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Figure Caption

Figure 1: Formulae of the nitrone spin traps **1-4** studied.

Figure 2: Formulae of 3CP **5**, used as internal reference, and of the spin adducts N-O_2H **6-9** studied.

Figure 3: EPR signal obtained in a pH 6.2 buffer by generating superoxide with a xanthine/xanthine oxidase system in the presence of TN **3** (0.03 mol dm^{-3}) and of 3CP **5** (0.6 10^{-6} mol dm^{-3}). The three lines of **5** spectrum are marked by downwards arrows. The other lines belong to the spectrum of TN-O_2H **7**. The framed part corresponds to the signal portion recorded to achieve the kinetic curves.

Figure 4: Experimental (full lines) and calculated (dotted lines) kinetic curves indicating the time-dependent changes in TN-O_2H **8** concentration. **A) 8** was produced at pH 6.2 by generating superoxide in the presence of: a) 5 mmol dm^{-3} **3**, b) 30 mmol dm^{-3} **3**, and c) 125 mmol dm^{-3} **3**. **B) 8** was produced at pH 9.3 by generating superoxide in the presence of: a) 20 mmol dm^{-3} **3**, b) 40 mmol dm^{-3} **3**, and c) 125 mmol dm^{-3} **3**. Calculated curves, obtained according to the model described by equations. (5)-(8), led to k_{Tap} and k_D values given in table 2.

Figure 5: Variation of the apparent rate constant k_{Tap} with pH for DEPO **2** (▼),EMPO **1** (●),TN **3** (⋆), and DMPO **4** (■). The curves have been calculated using equation (19) and the rate constant values listed in table 3. Experimental points have been obtained from k_{Tap} values listed in table 2.

Table 1. EPR hyperfine splitting constants for the nitroxides **5-9** in 0.1 mol dm^{-3} phosphate buffer (pH range: 6.2-9.3)

	a_N / mT	$a_{H\beta}$ / mT	other / mT
5	1.62	/	/
6	1.32	1.09	0.10 ($a_{H\gamma}$)
7	1.26	0.99	0.12 ($a_{H\gamma}$)
8	1.34	0.16	4.24 (a_P)
9	1.43	1.17	0.12 ($a_{H\gamma}$)

Table 2. Apparent rate constants for the spin trapping of superoxide by nitrones **1-4** (k_{Tap}) and for the decay of nitrone-superoxide spin adducts (k_D) at various pH.

nitrone	pH	k_{Tap} / dm^3 mol^{-1} s^{-1}	[N] / mmol dm^{-3}	k_D / 10^{-3} s^{-1}
EMPO **1**	6.2	101 ± 8	150	0.88 ± 0.01
			30	0.48± 0.01
			10	0.45± 0.01
	7.2	10.9 ± 0.1	200	1.25 ± 0.04
			30	0.65 ± 0.04
			10	0.60 ± 0.04
	8.0	2.02 ± 0.08	150	0.41 ± 0.01
			30	0.40 ± 0.01
			15	0.51± 0.01
	8.8	0.68 ± 0.05	150	2.66 ± 0.03
			30	3.68 ± 0.03
			10	3.56 ± 0.03
	9.3	0.60 ± 0.03	150	17.89 ± 0.78
			30	14.11 ± 0.78
			10	9.38 ± 0.78
DEPO **2**	6.2	185 ± 15	10	6.57 ± 0.29
			6.5	4.41± 0.29
	7.2	31 ± 1.3	15	2.06 ± 0.52
			5	1.04 ± 0.52
	8.0	3.54 ± 0.28	10	21.28 ± 0.48
			6	10.60 ± 0.48
	8.8	3.18 ± 0.32	5	6.26 ± 1.35
			3	3.18 ± 1.35
	9.3	1.98 ± 0.11	10	15.84 ± 1.81
			3	10.56 ± 1.81
TN **3**	6.2	58.26± 2.67	125	2.40 ± 0.07
			30	1.93 ± 0.07
			5	1.57 ± 0.07
	7.2	8.90 ± 0.58	140	2.70 ± 0.27
			72	2.60 ± 0.27
			5	1.90 ± 0.27
	8.0	1.56 ± 0.11	125	4.11 ± 1.0
			40	4.30 ± 1.0
			20	4.64± 1.0
	8.8	0.77 ± 0.09	125	5.07 ± 0.88
			30	3.85 ± 0.88
			15	2.99 ± 0.88
	9.3	0.56 ± 0.06	125	6.59 ± 0.55
			40	6.10 ± 0.55
			20	5.49 ± 0.55

Table 2 (continued). Apparent rate constants for the spin trapping of superoxide by nitrones **1-4** (k_{Tap}) and for the decay of nitrone-superoxide spin adducts (k_D) at various pH.

DMPO **4**	6.2	27.48 ± 1.95	125	0.48 ± 0.1
			40	0.66± 0.1
			10	0.75± 0.1
	6.8	9.65 ± 0.60	150	5.4 ± 1.8
			40	4.9 ± 1.8
			30	5.4 ± 1.8
	7.2	2.0 ± 0.3	125	9.0 ± 1.7
			40	8.2 ± 1.7
			20	7.9 ± 1.7
	8.0	0.52 ± 0.09	130	15.0 ± 2.5
			75	14.4 ± 2.5
			60	11.4 ± 2.5
	9.3	0.35 ± 0.04	150	17.7 ± 4.5
			50	9.1 ± 4.5
			30	9.2 ± 4.5

Table 3. Rate constants for the spin trapping of hydroperoxyl radical ($k_{HO_2\cdot}$) and of superoxide ($k_{O_2\cdot-}$) by nitrones **1-4**.

nitrone	$k_{HO_2\cdot}$ / dm^3 mol^{-1} s^{-1}	$k_{O_2\cdot-}$ / dm^3 mol^{-1} s^{-1}	$k_{HO_2\cdot}$ / $k_{O_2\cdot-}$
EMPO **1**	2206 ± 4	0.41 ± 0.04	$5.38\ 10^3$
DEPO **2**	4015 ± 30	1.68 ± 0.65	$2.39\ 10^3$
TN **3**	1264 ± 3	0.57 ± 0.03	$2.22\ 10^3$
DMPO **4**	600 ± 30	0.34 ± 0.4	$1.76\ 10^3$

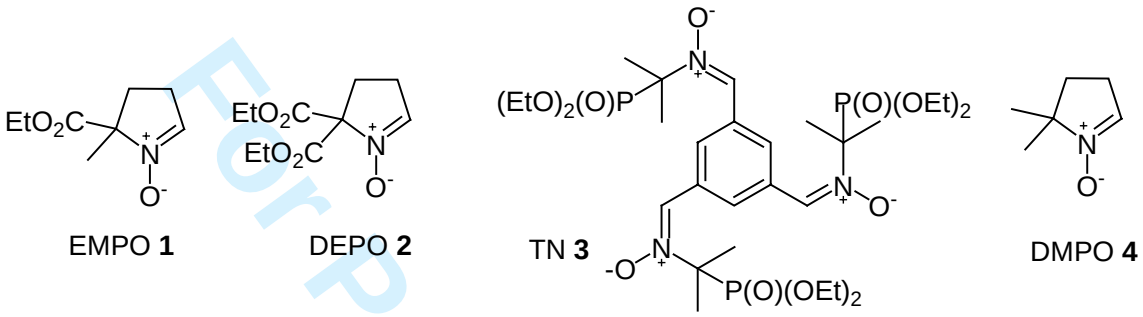

Figure 1 *A. Allouch* et al. *pH Effect on Superoxide Trapping by Nitrones*

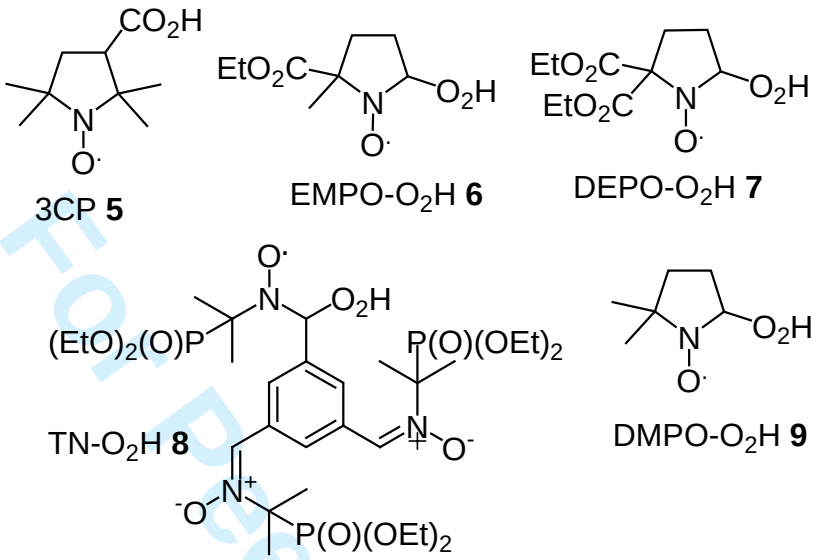

Figure 2 *A. Allouch* et al. *pH Effect on Superoxide Trapping by Nitrones*

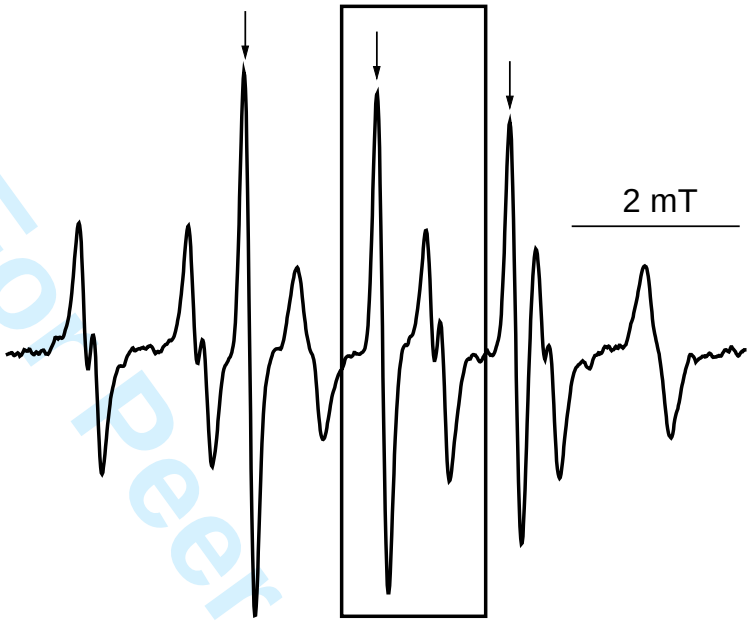

Figure 3 *A. Allouch* et al. *pH Effect on Superoxide Trapping by Nitrones*

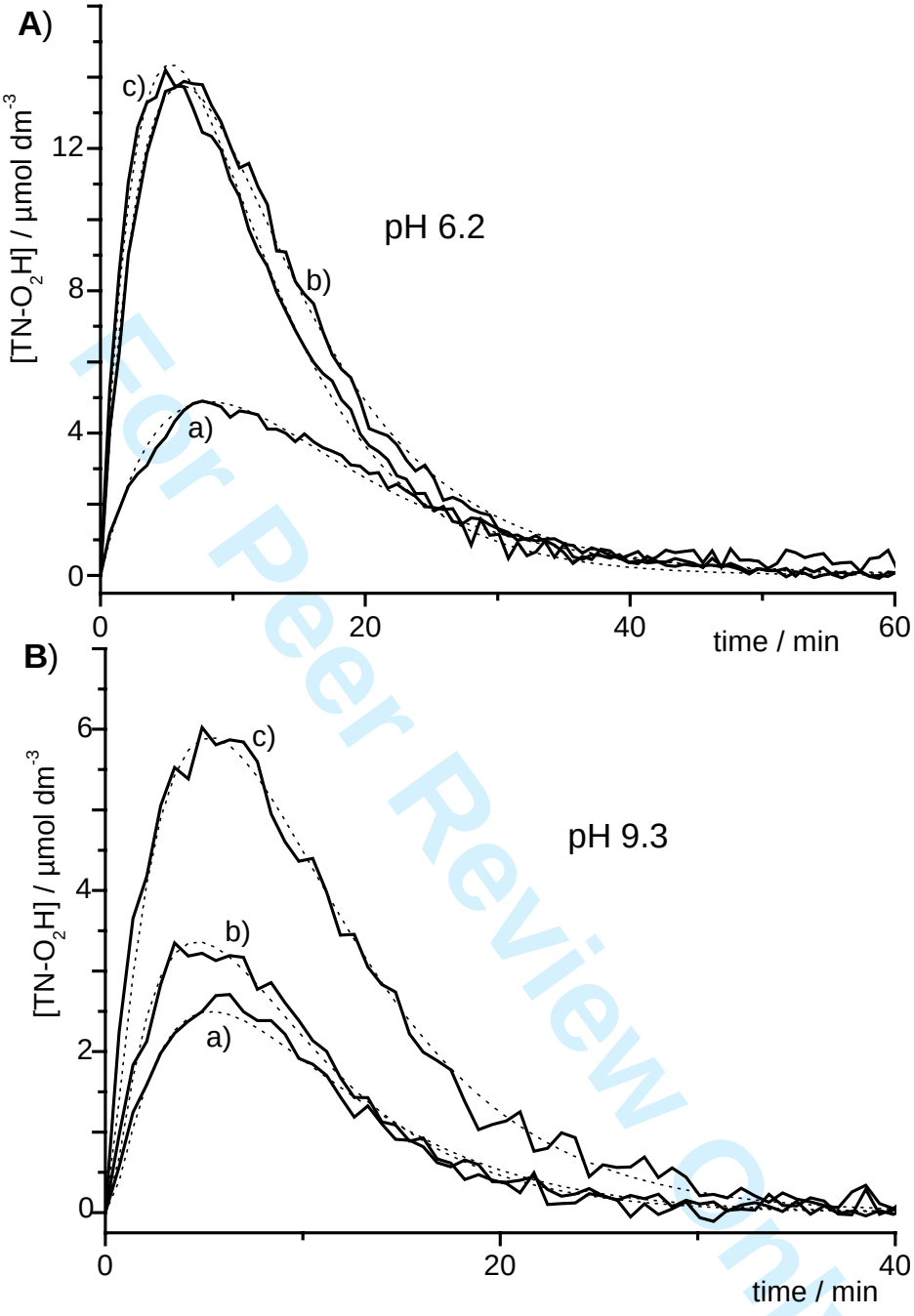

Figure 4 *A. Allouch* et al. *pH Effect on Superoxide Trapping by Nitrones*

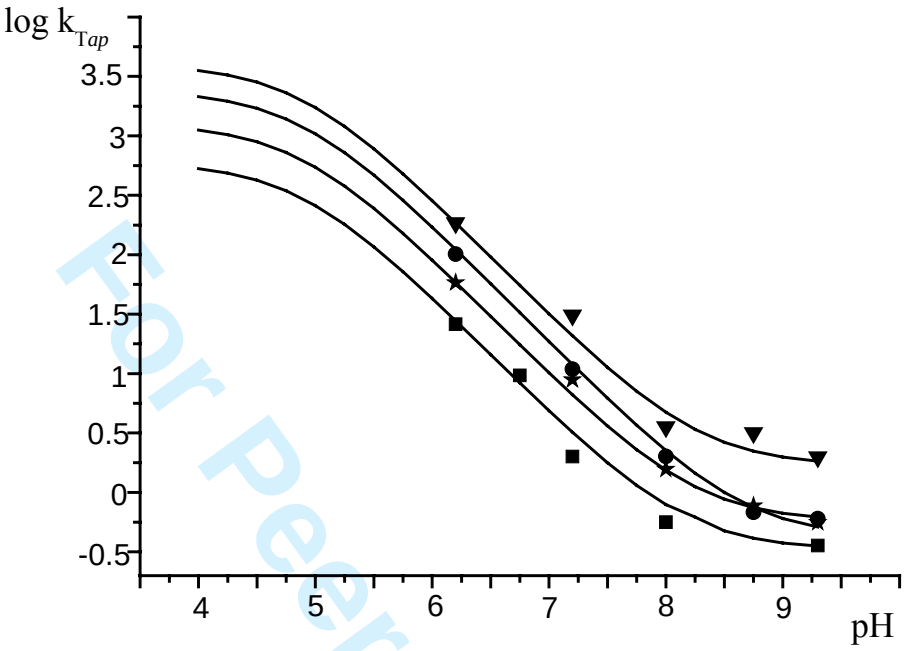

Figure 5 *A. Allouch* et al. *pH Effect on Superoxide Trapping by Nitrones*

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Effect of pH on superoxide/ hydroperoxyl radical trapping by nitrones: an EPR/kinetic study

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The pH dependence of the apparent rate constant k_{Tap} for $O_2^{\cdot-}/HO_2^{\cdot}$ trapping by four nitrones was examined. In each case, kinetic curves were obtained after treatment of EPR spectra of the spin adduct formed using both singular value decomposition and pseudo-inverse deconvolution methods. Modelling these curves led to evaluate k_{Tap} at various pH values. Analysis of the pH dependence of k_{Tap} permitted the determination of the rate constants for the spin trapping of $O_2^{\cdot-}$ and of $HO_2^{\cdot}$ separately. Whatever the nitrone, our results clearly show that the EPR signals of the nitrone/superoxide spin adducts observed in aqueous media were essentially due to the trapping of the protonated species $HO_2^{\cdot}$

Keywords: EPR; Kinetics; pH Effect; Spin Trapping; Superoxide; Nitrones

1 Introduction

Since the EPR/spin trapping technique was first introduced [1-3], it has found numerous applications in the detection of free radicals produced in chemistry, biology or medicine. It has been notably employed for detecting superoxide in aqueous media using nitrone spin traps [4-6]. For this purpose, a nitrone must trap rapidly this radical and must lead to a long-lived spin adduct. Several reliable methods have been described to determine k_D, the rate constant for the nitrone/superoxide spin adduct decay [7-13]. In contrast, evaluating the apparent rate of superoxide trapping by a nitrone, k_{Tap}, is more problematic and major disagreements exist regarding the data published in this field [11,14-21]. The most popular method used in these studies involves a competition between the nitrone and a superoxide scavenger, and implies that the superoxide spontaneous dismutation, the spin adduct decay and the consumption of the competitor during the course of the experiment correspond to negligible events [11,14-17]. Two years ago, we brought evidences of the importance of these three reactions and showed that their omission resulted in significantly overestimating k_{Tap} [22]. Another method based on a competition between the superoxide trapping by the nitrone and the spontaneous dismutation of this radical yielded lower k_{Tap} values []18,19]. This prompted us to elaborate a new kinetic approach to the evaluation of k_{Tap} in buffered media [20,21]. It was also based on a competition between the superoxide trapping and its spontaneous dismutation, and kinetic curves were obtained after treatment of EPR spectra using both singular value decomposition (SVD) and pseudo-inverse deconvolution methods before their computer modelling. Whatever the method used, it is widely admitted that the kinetic parameters related to the spin trapping of superoxide by nitrones are pH dependent. On one hand, nitrone/superoxide spin adducts decayed

more slowly in acidic media [7-9]. On the other hand, k_{Tap} also greatly varied with pH [14,16], owing to the fact that superoxide $O_2^{-\cdot}$ and its protonated form $HO_2^{\cdot}$ were not distinguished in these studies, both radicals leading to the same EPR-detectable spin adduct in aqueous media. The relative concentrations of $HO_2^{\cdot}$ and of $O_2^{-\cdot}$ depend on pH, the pK_A value for the acidic dissociation reaction of the hydroperoxyl radical being equal to 4.88 [23]. Considering that nitrones react differently with $HO_2^{\cdot}$ and $O_2^{-\cdot}$, this justifies the pH dependence of k_{Tap} .

Recently, we have found that three nitrones were particularly efficient for superoxide detection: the ethyl 2-methyl-3,4-dihydro-*2H*-pyrrole-carboxylate *1*-oxide **1** (EMPO, $k_{Tap} \approx 11$ $dm^3mol^{-1}s^{-1}$ at pH 7.2), the diethyl 3,4-dihydro-2H-pyrrole-2,2-dicarboxylate *1*-oxide **2** (DEPO, $k_{Tap} \approx 31$ $dm^3mol^{-1}s^{-1}$ at pH 7.2), and 1,3,5-tri [(N- (1-diethylphosphono)-1-methyl-ethyl) -*N*-oxy-aldimine] benzene **3** (TN, $k_{Tap} \approx 9$ $dm^3mol^{-1}s^{-1}$ at pH 7.2) [20,21]. Among them, TN is the only lipophilic trap, with an octanol/water partition coefficient higher than 300 [24]. By far, DEPO was found the fastest at trapping superoxide, but the spin adduct thus formed decayed very rapidly, particularly when high nitrone concentrations were used. However, kinetic experiments with all these traps were only performed at pH 7.2. To proceed with this study, we have examined the pH dependence of the rate constant for the superoxide trapping by EMPO, DEPO, TN and have compared the results obtained to that given by the well-known 2,2-dimethyl-3,4-dihydro-*2H*-pyrrole *1*-oxide **4** (DMPO) (see figure 1). Analysis of the data obtained at various pH permitted to evaluate separately the rate constant for the trapping of $HO_2^{\cdot}$ and $O_2^{-\cdot}$ by the nitrone considered and to determine which species is actually responsible for the formation of the adduct detected by EPR.

[Insert figure 1 about here]

In this whole study, the spin adduct detected by EPR after trapping either $O_2^{-\cdot}$ or $HO_2^{\cdot}$ with a nitrone N in aqueous media will be denoted N-O_2H. With the aim of simplifying the notation, the term 'superoxide' will first be used to designate both superoxide and hydroperoxyl radicals as a whole in the part of this work devoted to the study of the pH effect on k_{Tap}. Similarly, the symbol '$O_2^{-\cdot}$' in the kinetic model [equations (1)-(4)] as well as in the rate equations (5)-(8) will represent either protonated or non-protonated forms of the radical. In a second part of this study, the two species will be considered separately to evaluate each individual contribution to the formation of N-O_2H.

2 Experimentals

2.1 Materials

The nitrones EMPO **1**, DEPO **2** and TN **3** were synthesised and purified according to procedures described previously [24-27]. DMPO **4** (Sigma-Aldrich Co.) was purified by vacuum distillation before use. Diethylenetriaminepentacetic acid (DTPA), 3-carboxy-2,2,5,5-tetramethylpyrrolidin-1-oxyl **5** (3CP), xanthine and xanthine oxidase were obtained from Sigma-Aldrich Co. Buffer solutions, were stirred gently for six hours in the presence of a chelating iminodiacetic acid resin to remove trace metal impurities.

2.2 Achievement of experimental kinetic curves

All experiments were made in 0.1 mol dm^{-3} phosphate buffer (pH range: 6.2-9.3). Superoxide was produced using a xanthine-xanthine oxidase system. In a standard experiment, the medium contained a nitrone N (3-200 mmol. dm^{-3}), 1.6 mmol.dm^{-3} xanthine, 3 mmol.dm^{-3} diethylenetriaminepentacetic acid, 3CP (0.5-1.1 µmol dm^{-3}) used as internal standard, and 0.04 unit.cm^{-3} xanthine oxidase. Air was bubbled into the

medium for one min. before addition of xanthine oxidase. A part of the EPR signal showing at least one line of the superoxide adduct N-O_2H and one line of 3CP spectra was recorded every 42 s. Noise was reduced using the SVD procedure, and the kinetic curves (adduct concentration *vs*. time) were obtained after deconvolution of the signal using the pseudo-inverse method, according to procedures extensively described previously [20]. These calculations were achieved with the help of a home-made computer programme written in FORTRAN, using subroutines given in Numerical Recipes [28]. At each pH value and for each nitrone, three experiments were performed at three different nitrone concentrations, one of which was set high enough to trap out most of the superoxide produced. In the case of DEPO, the procedure was modified as described previously [20,21], because the spin adduct decays too rapidly at high nitrone concentrations. EPR essays were carried out at 20°C in capillary tubes by using a Bruker EMX spectrometer operating at X-band with 100 kHz modulation frequency. General instrument settings were as follows: microwave power, 20 mW; modulation amplitude, 0.1-0.18 mT; receiver gain, 2 10^5 - 2 10^6; time constant, 164 ms; scan time, 21 s; scan width, 1.5 - 6 mT; 2 scans.

2.3 Determination of the apparent rate constant k_{Tap}

At a given pH, the kinetic model considered can be described by equations (1)-(4),

$$X \xrightarrow{k_X} O_2^{\cdot -} \qquad (1)$$

$$2\ O_2^{\cdot -} + 2\ H^+ \xrightarrow{k_{dis}} O_2 + H_2O_2 \qquad (2)$$

$$O_2^{\cdot -} + H^+ + N \xrightarrow{k_{Tap}} N\text{-}O_2H \qquad (3)$$

$$N\text{-}O_2H \xrightarrow{k_D} Y \qquad (4)$$

where k_X and k_D are the rate constants for the generation of superoxide and for the decay of the spin adduct N-O_2H, respectively, Y representing EPR silent products and X an intermediate derived from xanthine [20]. The apparent rate constant for the second-order trapping reaction is denoted k_{Tap}. The rate equations (5)-(8) can be written from this kinetic model:

$$d[X]/dt = -k_X [X] \quad (5)$$

$$d[O_2^{\cdot-}]/dt = k_X [X] - k_{Tap} [N] [O_2^{\cdot-}] - 2 k_{dis} [O_2^{\cdot-}]^2 \quad (6)$$

$$d[N\text{-}O_2H]/dt = k_{Tap} [N] [O_2^{\cdot-}] - k_D [N\text{-}O_2H] \quad (7)$$

$$d[N]/dt = - k_{Tap} [N] [O_2^{\cdot-}] \quad (8)$$

The term 'superoxide' and the symbol '$O_2^{\cdot-}$' designate here the protonated and non-protonated forms of the radical indiscriminately. The apparent rate constant for the trapping reaction by the nitrone N, k_{Tap}, is pH dependent and includes the contribution of both $HO_2^{\cdot}$ and $O_2^{\cdot-}$ trapping. The second order rate constant for the superoxide spontaneous dismutation reaction, k_{dis}, is also pH-dependent. Computer modelling of the kinetic curves obtained was achieved using a home-made programme and according to equations (5)-(8). In these calculations, the nitrone initial concentration is an experimental parameter, the initial concentrations of superoxide and of N-O_2H are equal to zero, and k_{dis} was evaluated as described further. The standard least-square method was applied to fit the experimental curves, yielding the other parameters. Therefore, the three kinetic curves obtained at three different nitrone concentrations were considered jointly and modelled with the same parameter set (except for parameters varying with the nitrone concentration). The values obtained for the initial concentration $[X]_0$ and for the rate constant k_X had no real meaning and only came up as an empirical model of the superoxide generation. Since they may vary with the solutions of either xanthine or

xanthine oxidase used [20], experiments at various nitrone concentrations were performed with exactly the same superoxide generator. The model corresponding to equations (9)-(12), which was first established by Baxendale [29], describes the superoxide spontaneous dismutation in aqueous media. Here, reaction (12) is negligible below pH 9.5.

$$HO_2^{\cdot} \underset{}{\overset{K_A}{\rightleftharpoons}} O_2^{-\cdot} + H^+ \qquad (9)$$

$$2\ HO_2^{\cdot} \xrightarrow{k_1} H_2O_2 + O_2 \qquad (10)$$

$$O_2^{-\cdot} + HO_2^{\cdot} \xrightarrow{k_2} H_2O_2 + O_2 + HO^- \qquad (11)$$

$$2\ O_2^{-\cdot} \xrightarrow{k_3} H_2O_2 + O_2 + 2\ HO^- \qquad (12)$$

The rate constant k_{dis} can be determined at any pH from equations (13)-(14), where pK_A, k_1 and k_2 are equal to 4.88, 76 10^4 $dm^3.mol^{-1}.s^{-1}$ and 85 10^6 $dm^3.mol^{-1}.s^{-1}$, respectively [23].

$$k_{dis} = (k_1 + \chi\ k_2) / (1 + \chi)^2 \qquad (13)$$

$$\chi = K_A / [H^+] = 10^{(pH-pK_A)} \qquad (14)$$

2.3 Determination of the trapping rate constants $k_{HO_2\cdot}$ and $k_{O_2^{-}\cdot}$.

Because nitrones react differently with $O_2^{-\cdot}$ and with $HO_2^{\cdot}$, reactions (15) and (16) should be considered instead of reaction (3) in the kinetic model, $k_{HO_2\cdot}$ and $k_{O_2^{-}\cdot}$ being the rate constants for the trapping of superoxide radical anion ($O_2^{-\cdot}$) and hydroperoxyl radical ($HO_2^{\cdot}$), respectively, by the nitrone N.

$$O_2^{-\cdot} + N + H^+ \xrightarrow{k_{O_2^{-}\cdot}} N\text{-}O_2H \qquad (15)$$

$$HO_2^{\cdot} + N \xrightarrow{k_{HO_2\cdot}} N\text{-}O_2H \qquad (16)$$

According to Finkelstein *et al.* [14], equation. (17) can be written from equations (14)-(16).

$$k_{Tap} = (k_{HO_2\cdot} + \chi\, k_{O_2\cdot -}) / (1 + \chi) \qquad (17)$$

Using the method described in the previous section, k_{Tap} was determined for each nitrone at five pH values ranging from 6.2 to 9.3. The experimental curves thus obtained were modelled using equation (17), which led to $k_{HO_2\cdot}$ and $k_{O_2\cdot -}$.

3 Results and discussion

3.1 Determination of the rate constants at various pH

Experiments were performed in order to evaluate the pH influence on the trapping of superoxide by four nitrones: EMPO **1**, DEPO **2**, TN **3** and DMPO **4**. Whatever the species trapped by the nitrone N ($O_2^{\cdot -}$ or $HO_2^{\cdot}$, both designated here by the term 'superoxide'), only the protonated adduct N-O_2H will be detected by EPR. Actually, the anionic radical species N-O_2^- is not EPR silent, but its concentration in the pH range considered is negligible, considering the high pKa values of hydroperoxides.

The formulae of the stable nitroxide **5** and of the spin adducts **6-9** considered in this study have been drawn in figure 2. Before each kinetic experiment, a full EPR spectrum of the medium was recorded. All the spectra thus obtained were analysed using the computer program elaborated by Duling [30], which led to the values listed in table 1 for the hyperfine splitting constants (hfsc*s*) with the nitrogen (a_N), the β-hydrogen ($a_{H\beta}$) and eventually a γ-hydrogen ($a_{H\gamma}$) or a β-phosphorus (a_P) nuclei. These hfsc*s* were not found to vary in the pH range considered and agree with the literature data [4,10,24,27]. In a previous study [24], we have shown that

the mono-nitroxide **8** (i.e. the mono-adduct) was almost exclusively formed at the tri-nitrone TN **3** concentrations used, the proportion of bi-nitroxide being negligible.

[Insert figure 2 and table 1 about here]

The kinetic approach used, based on a competition between the superoxide trapping and its spontaneous dismutation at a given pH value, permits the consideration of the whole kinetic curve of superoxide adduct formation and decay. This method has been extensively described elsewhere and will only be briefly explained here [20]. For each nitrone N considered and at each pH value studied, a part of the EPR spectrum of N-O_2H was recorded as a function of time at various nitrone concentrations, in the presence of an internal reference (see experimental section). In order to illustrate the method employed, an EPR spectrum of the adduct TN-O_2H **8** recorded at pH 6.2 in the presence of the internal standard 3CP is shown in figure 3, the framed part corresponding to the signal range chosen to perform the kinetic experiments. Using both singular value decomposition and pseudo-inverse deconvolution methods, kinetic curves indicating the time dependant changes in the N-O_2H concentration at various pH were achieved. As an example, the curves obtained with the spin trap **3** at pH 6.2 and at pH 9.3 are given in figure 4. Using equations. (5)-(8), computer modelling of the experimental kinetic curves obtained with each nitrone at each pH considered yielded the values of k_D and k_{Tap} listed in table 2.

[Insert figures 3 and 4 and table 2 about here]

It has been shown that the rate constant for the first-order decay of nitrone/superoxide spin adducts increased with pH and with the spin trap concentration [7-9,20,21,30]. Overall, the values listed for k_D in table 2 agree with

these observations, though a few deviations from this general trend can be noticed. These could be explained by considering two main factors. Firstly, EPR signals recorded under certain extreme conditions (e.g. at high pH values) were often very weak. Actually, it would have been impossible to achieve the kinetic curves from these spectra without the help of SVD and deconvolution methods. In this case, k_D determination could be less accurate, as shown for instance by the errors given in table 2. Secondly, the first-order decay of nitrone/superoxide spin adducts in aqueous media are known to depend on several parameters other than pH and nitrone concentration. In particular, minor changes in the superoxide generator (i.e. in solutions of xanthine or of xanthine oxidase) may affect significantly N-O_2H life-time [8,10,20,21]. Considering the number of experiments performed in this study, different solutions of xanthine and of xanthine oxidase have been used, thereby rending k_D evaluation less accurate. The dependence of the decay rate of nitrone/superoxide adducts on various parameters (pH, nitrone concentration, superoxide generating system, etc.) is certainly an interesting problem that would warrant a more thorough study. A better knowledge of the mechanisms responsible for N-O_2H decay in aqueous solutions would permit the design of new spin traps with increased superoxide adduct life-time. However, the purpose of the present work was mainly to scrutinise the effect of pH on the superoxide trapping rate. Note that the three series of spectra at the three different nitrone concentrations, for each nitrone and at each pH, were recorded under exactly the same conditions. Thus, in the kinetic approach used, minor changes in the superoxide generator would not affect k_{Tap} determination.

A fast survey of k_{Tap} values given in table 2 shows that the same nitrone ranking was obtained at each pH considered: DEPO was always found the fastest at trapping superoxide, followed by EMPO, TN and DMPO. Whatever the nitrone, our results confirmed that k_{Tap} diminished at higher pH. This could suggest that the nitrones would trap the hydroperoxyl radical more rapidly than the superoxide radical anion. At this point however, interpretation of the experimental results requires an more advanced analysis, in which contributions of the protonated and non protonated radicals should be separated.

3.2 Analysis of k_{Tap} variation with pH

In this last part of our study, the superoxide radical anion and the protonated hydroperoxyl radical were distinguished. Our purpose was to determine in which extent each of these two species contributes to the N-O_2H EPR signal observed. As mentioned in the experimental section, $k_{O_2^{-\cdot}}$ and $k_{HO_2^{\cdot}}$ are the second-order rate constants for the trapping of $O_2^{-\cdot}$ and of $HO_2^{\cdot}$ by a nitrone N, respectively. Equation (17), which can be rewritten as equation (18), indicates the relation between $k_{O_2^{-\cdot}}$, $k_{HO_2^{\cdot}}$ and k_{Tap}. Equation(19) can be written from equation (18), in which pK_A is equal to 4.88.

$$k_{Tap} = [k_{HO_2^{\cdot}} + 10^{(pH-pK_A)}\, k_{O_2^{-\cdot}}] / [1 + 10^{(pH-pK_A)}] \qquad (18)$$

$$\log k_{Tap} = \log\, [k_{HO_2^{\cdot}} + (pH - 4.88)\, k_{O_2^{-\cdot}}] - pH + 4.88 \qquad (19)$$

Fitting the model described by equation (18) to the experimental points for nitrones **1-4** yielded the values reported in table 3 for the rate constants $k_{O_2^{-\cdot}}$ and $k_{HO_2^{\cdot}}$. . In figure 5, the decimal logarithm of k_{Tap} has been plotted *vs*. pH. The curves have been obtained according to equation (19) and using the rate constant values listed in

table 3, showing for each nitrone a rather good fit between experimental and calculated k_{Tap} values.

[Insert figure 5 about here]

In the particular case of DMPO, $k_{O_2^{-\cdot}}$ was found near nil, considering the error in its determination. This last result could seem in contradiction with that published by Goldstein *et al.* [32], who have found that $k_{O_2^{-\cdot}}$ was equal to 170 ± 40 $dm^3\ mol^{-1}s^{-1}$ for DMPO. However, they obtained this value by measuring the kinetics of $O_2^{-\cdot}$ consumption in the presence of **4** using the pulse radiolysis technique, while we followed the kinetics of formation of **9** using EPR spectroscopy. According to Goldstein *et al.* [32], only a fraction of the superoxide radical anion yields the nitroxide **9**. This explains the discrepancy mentioned above, all the more so since the yield of the reaction: **4** + $O_2^{-\cdot}$ → **9** is unknown. Consequently, the EPR method is obviously more appropriate to kinetic studies of superoxide trapping by nitrones.

Whatever the nitrone, there is always three order of magnitude between $k_{HO_2^{\cdot}}$ and $k_{O_2^{-\cdot}}$ (see table 3). Thus, the trapping of $O_2^{-\cdot}$ does not intervene significantly in the N-O_2H formation until the superoxide concentration is much higher than that of hydroperoxyl radical. For instance, the proportion of the EPR signal of **6** originating from the trapping of $O_2^{-\cdot}$ by EMPO is lower than 1% at pH 6.5, than 2.5% at pH 7 and than 8% at pH 7.5. In the case of DMPO **4**, these values are raised to *ca.* 2.5%, 8% and 25% at pH 6.5, 7 and 7.5 respectively. This means that the EPR signals of the spin adducts N-O_2H observed in aqueous media around neutral pH were almost exclusively due to the trapping of the protonated species $HO_2^{\cdot}$

4 Conclusion

This study confirms that the spin trapping of $O_2^{\cdot-}/HO_2^{\cdot}$ by nitrones is overall more efficient at low pH. Whatever the nitrone N, the formation of N-O_2H is not only faster in acidic media but the decay rate of this spin adduct increases with pH. When it comes to the trapping rate, the same nitrone ranking was obtained at each pH studied: DEPO was found the fastest at trapping superoxide, followed by EMPO, and TN, these three compounds being much more efficient than DMPO. Examining the pH dependence of the kinetics of N-O_2H formation permitted to evaluate separately the rate constant values for the spin trapping of superoxide radical anion and of hydroperoxyl radical by four nitrones. The results obtained with **1-4** clearly showed that the EPR signals of the nitrone/superoxide spin adducts observed in aqueous media were essentially due to the trapping of the protonated species $HO_2^{\cdot}$. For the nitrones tested, $k_{O_2^{\cdot-}}$ was always found lower than 2 dm^3 mol^{-1} s^{-1}. In previous studies conducted with ten commonly used nitrones, **1-3** were found the most efficient for superoxide/hydroperoxyl radical trapping at neutral pH [20-21]. Thus, this very low reactivity of nitrones with the superoxide radical anion should be considered as a serious drawback, precluding the use of these compounds in quantitative studies of $O_2^{\cdot-}/HO_2^{\cdot}$ formation in aqueous media. Despite this, compounds **1-3** could still find interesting applications in qualitative studies in water or biological media. Therefore, the development of new and more efficient traps is still a necessity, in particular for superoxide radical anion detection.

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Figure Caption

Figure 1: Formulae of the nitrone spin traps **1-4** studied.

Figure 2: Formulae of 3CP **5**, used as internal reference, and of the spin adducts N-O_2H **6-9** studied.

Figure 3: EPR signal obtained in a pH 6.2 buffer by generating superoxide with a xanthine/xanthine oxidase system in the presence of TN **3** (0.03 mol dm^{-3}) and of 3CP **5** (0.6 10^{-6} mol dm^{-3}). The three lines of **5** spectrum are marked by downwards arrows. The other lines belong to the spectrum of TN-O_2H **7**. The framed part corresponds to the signal portion recorded to achieve the kinetic curves.

Figure 4: Experimental (full lines) and calculated (dotted lines) kinetic curves indicating the time-dependent changes in TN-O_2H **8** concentration. **A) 8** was produced at pH 6.2 by generating superoxide in the presence of: a) 5 mmol dm^{-3} **3**, b) 30 mmol dm^{-3} **3**, and c) 125 mmol dm^{-3} **3**. **B) 8** was produced at pH 9.3 by generating superoxide in the presence of: a) 20 mmol dm^{-3} **3**, b) 40 mmol dm^{-3} **3**, and c) 125 mmol dm^{-3} **3**. Calculated curves, obtained according to the model described by equations. (5)-(8), led to k_{Tap} and k_D values given in table 2.

Figure 5: Variation of the apparent rate constant k_{Tap} with pH for DEPO **2** (▼),EMPO **1** (●),TN **3** (★), and DMPO **4** (■). The curves have been calculated using equation (19) and the rate constant values listed in table 3. Experimental points have been obtained from k_{Tap} values listed in table 2.

Table 1. EPR hyperfine splitting constants for the nitroxides **5-9** in 0.1 mol dm^{-3} phosphate buffer (pH range: 6.2-9.3)

	a_N / mT	$a_{H\beta}$ / mT	other / mT
5	1.62	/	/
6	1.32	1.09	0.10 ($a_{H\gamma}$)
7	1.26	0.99	0.12 ($a_{H\gamma}$)
8	1.34	0.16	4.24 (a_P)
9	1.43	1.17	0.12 ($a_{H\gamma}$)

Table 2. Apparent rate constants for the spin trapping of superoxide by nitrones **1-4** (k_{Tap}) and for the decay of nitrone-superoxide spin adducts (k_D) at various pH.

nitrone	pH	k_{Tap} / dm^3 mol^{-1} s^{-1}	[N] / mmol dm^{-3}	k_D / 10^{-3} s^{-1}
EMPO **1**	6.2	101 ± 8	150	0.88 ± 0.01
			30	0.48± 0.01
			10	0.45± 0.01
	7.2	10.9 ± 0.1	200	1.25 ± 0.04
			30	0.65 ± 0.04
			10	0.60 ± 0.04
	8.0	2.02 ± 0.08	150	0.41 ± 0.01
			30	0.40 ± 0.01
			15	0.51± 0.01
	8.8	0.68 ± 0.05	150	2.66 ± 0.03
			30	3.68 ± 0.03
			10	3.56 ± 0.03
	9.3	0.60 ± 0.03	150	17.89 ± 0.78
			30	14.11 ± 0.78
			10	9.38 ± 0.78
DEPO **2**	6.2	185 ± 15	10	6.57 ± 0.29
			6.5	4.41± 0.29
	7.2	31 ± 1.3	15	2.06 ± 0.52
			5	1.04 ± 0.52
	8.0	3.54 ± 0.28	10	21.28 ± 0.48
			6	10.60 ± 0.48
	8.8	3.18 ± 0.32	5	6.26 ± 1.35
			3	3.18 ± 1.35
	9.3	1.98 ± 0.11	10	15.84 ± 1.81
			3	10.56 ± 1.81
TN **3**	6.2	58.26± 2.67	125	2.40 ± 0.07
			30	1.93 ± 0.07
			5	1.57 ± 0.07
	7.2	8.90 ± 0.58	140	2.70 ± 0.27
			72	2.60 ± 0.27
			5	1.90 ± 0.27
	8.0	1.56 ± 0.11	125	4.11 ± 1.0
			40	4.30 ± 1.0
			20	4.64± 1.0
	8.8	0.77 ± 0.09	125	5.07 ± 0.88
			30	3.85 ± 0.88
			15	2.99 ± 0.88
	9.3	0.56 ± 0.06	125	6.59 ± 0.55
			40	6.10 ± 0.55
			20	5.49 ± 0.55

Table 2 (continued). Apparent rate constants for the spin trapping of superoxide by nitrones **1-4** (k_{Tap}) and for the decay of nitrone-superoxide spin adducts (k_D) at various pH.

DMPO **4**	6.2	27.48 ± 1.95	125	0.48 ± 0.1
			40	0.66± 0.1
			10	0.75± 0.1
	6.8	9.65 ± 0.60	150	5.4 ± 1.8
			40	4.9 ± 1.8
			30	5.4 ± 1.8
	7.2	2.0 ± 0.3	125	9.0 ± 1.7
			40	8.2 ± 1.7
			20	7.9 ± 1.7
	8.0	0.52 ± 0.09	130	15.0 ± 2.5
			75	14.4 ± 2.5
			60	11.4 ± 2.5
	9.3	0.35 ± 0.04	150	17.7 ± 4.5
			50	9.1 ± 4.5
			30	9.2 ± 4.5

Table 3. Rate constants for the spin trapping of hydroperoxyl radical ($k_{HO_2\cdot}$) and of superoxide ($k_{O_2\cdot-}$) by nitrones **1-4**.

nitrone	$k_{HO_2\cdot}$ / $dm^3\ mol^{-1}\ s^{-1}$	$k_{O_2\cdot-}$ / $dm^3\ mol^{-1}\ s^{-1}$	$k_{HO_2\cdot} / k_{O_2\cdot-}$
EMPO **1**	2206 ± 4	0.41 ± 0.04	$5.38\ 10^3$
DEPO **2**	4015 ± 30	1.68 ± 0.65	$2.39\ 10^3$
TN **3**	1264 ± 3	0.57 ± 0.03	$2.22\ 10^3$
DMPO **4**	600 ± 30	0.34 ± 0.4	$1.76\ 10^3$

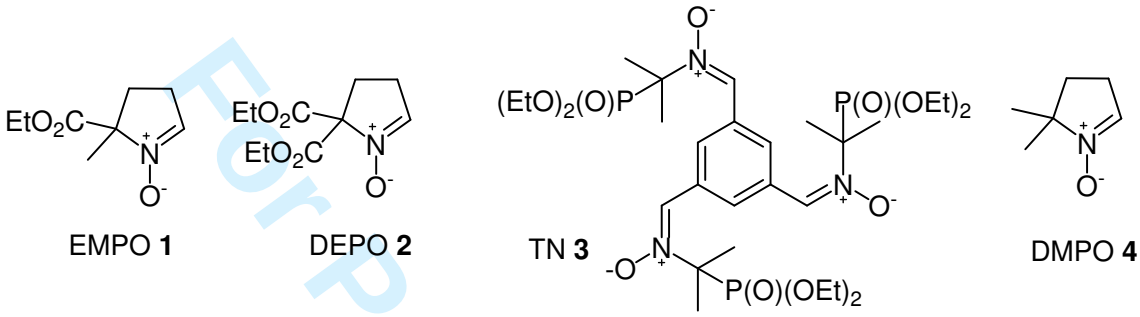

Figure 1 *A. Allouch* et al. *pH Effect on Superoxide Trapping by Nitrones*

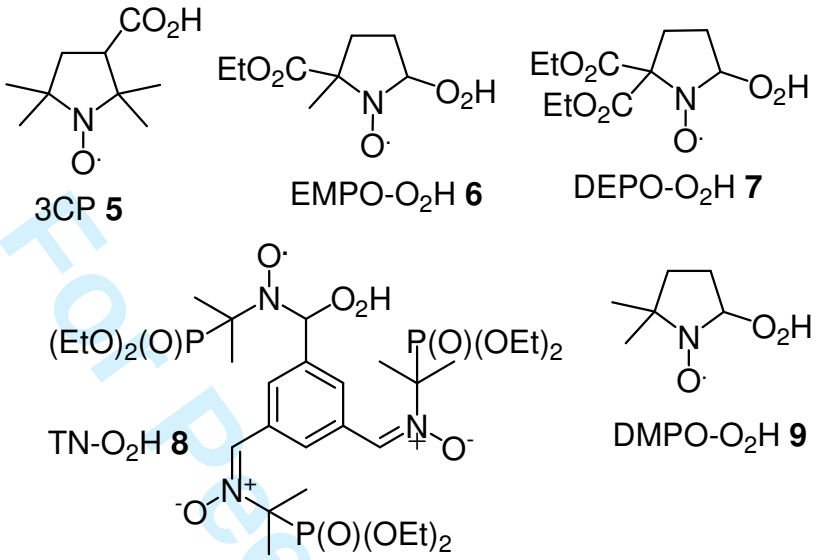

Figure 2 *A. Allouch* et al. *pH Effect on Superoxide Trapping by Nitrones*

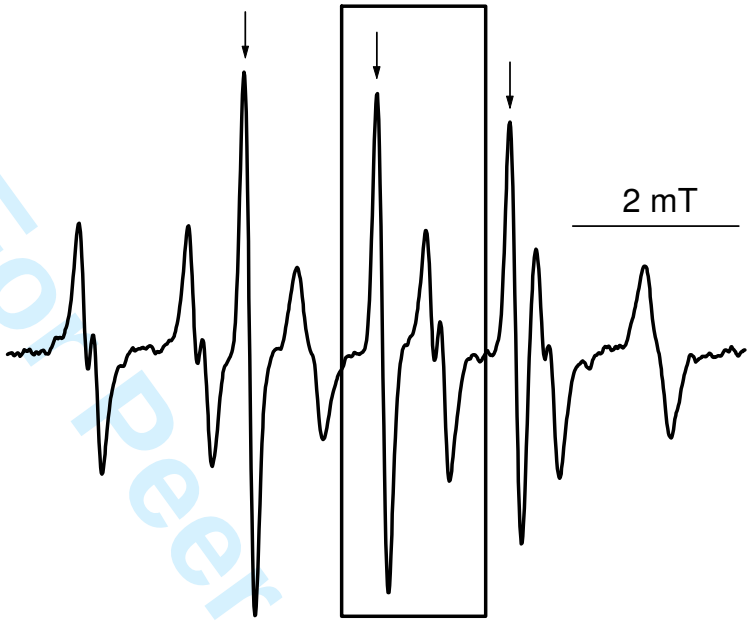

Figure 3 *A. Allouch* et al. *pH Effect on Superoxide Trapping by Nitrones*

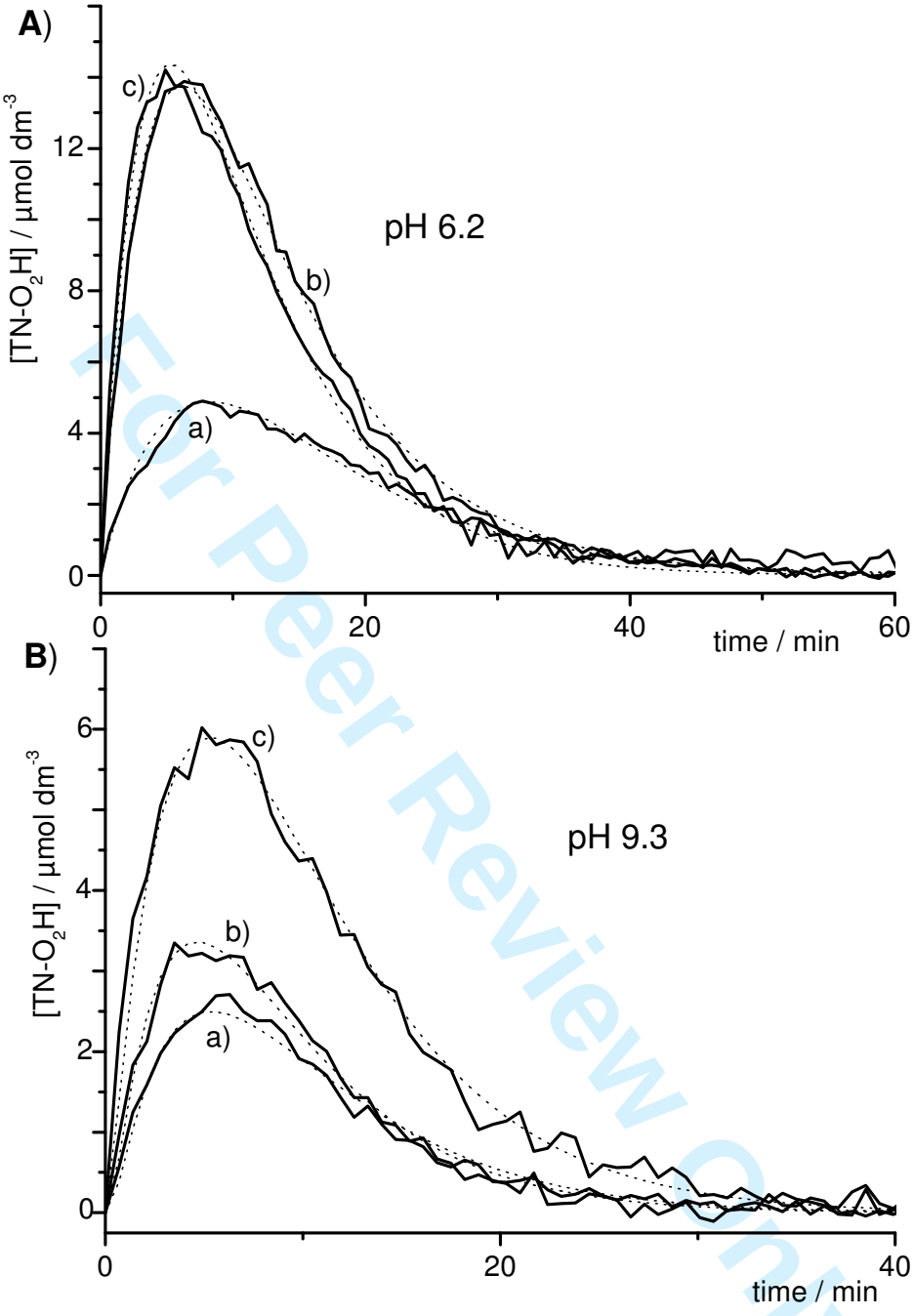

Figure 4 *A. Allouch* et al. *pH Effect on Superoxide Trapping by Nitrones*

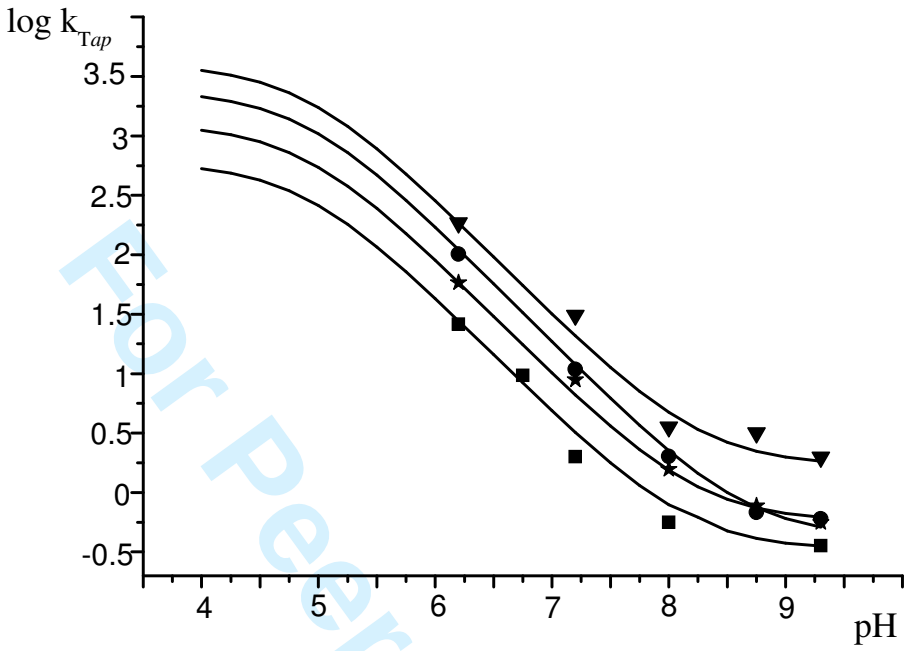

Figure 5 *A. Allouch* et al. *pH Effect on Superoxide Trapping by Nitrones*